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STUDIES OF WOUND HEALING IN THE PRESENCE OF AN ANGIOGENESIS FACTOR

Annual Report

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Basic fibroblast growth factor (bFGF) was found to increase wound healing in a healing-impaired model; the db/db mouse. When bFGF was applied to 6 mm punch biopsy wounds, a dramatic increase in granulation tissue formation was observed. bFGF-treated wounds appeared very similar to control normal wounds in respect to dermal thickness, compactness of dermis, numbers of migratory cells and number of capillaries. No effect on the epidermis was observed. We conclude, therefore, that bFGF may be a useful modality for the treatment of wounds in healing-impaired or healing-compromised patients.

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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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Introduction

During the past year, we explored the ability of basic fibroblast growth factor (bFGF) to stimulate wound healing in a healing impaired model using db/db mice. When compared to their heterozygous litter mates, these mice show a pronounced impairment of dermal healing. bFGF was found to increase the rate of wound healing in these mice so that little difference was observed between the control normal mice and experimental mice treated with bFGF. The parameters explored were dose of bFGF, number of applications, time course of healing, tensile strength, and specificity of the reaction.

Animals , - Female mutant diabetic mice, C57BL/ksJ db/db, and their littermates (db/+) were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a standard laboratory diet and water ad libitum, and used at 8 weeks age. Before the experiments, they were individually housed and checked for urinary glucose by reagent strips (Miles Laboratories Inc., Elkhart, IN).

Preparation of Reagents. - Recombinant bFGF was a gift from Synergen, Inc., Boulder CO. Carboxy Methyl Cellulose (MW .250 Kd) was purchased from Polysciences Inc. (Niles, IL). The vehicle solution consisted of 1.5% carboxy methylcellulose and 0.5% mouse serum in sterilized phosphate buffered saline solution. Various concentrations of bFGF solution were prepared by adding concentrated recombinant bFGF to the vehicle solution. Polyclonal antibodies against recombinant bFGF were raised in rabbits as described (Joseph-Silverstein et al., 1988) and IgG fractions were prepared by protein A-Sepharose column chromatography and lyophylized.

Wounding. - Mice were anesthetized with sodium pentobarbitol solution (40 mg/kg, intraperitoneally) and their dorsal hair was clipped. Two full thickness rounded wounds were prepared vertically on the back of each mouse

with a punch biopsy instrument (6 mm diameter, George Tiemann and Co., Long Island City, NY). After the operation, 20 ul of bFGF or vehicle solution was applied. A single mouse received the same test solutions in each wound to prevent possible effects from leakage do to one wound to the other. Once the wound was almost dry, the mice were allowed to recover from the anesthesia. The wounds were kept open during the experiment. In some experiments, mice were given reagents once a day until the fifth day.

Sample Preparation and Histological Evaluation. - On the indicated day, the mice were sacrificed by cervical dislocation using care not to pull back the skin. The wounds were excised and fixed in 10% buffered formalin solution. After overnight fixation, the tissue was trimmed and then cut through at the widest margin. The tissue was embedded in parafin and sectioned in 5 um. Six sections were placed on a slide, and stained with hematoxylin and eosin.

Histological evaluation was done in a blind way. Of the six sections in any one slide, the section with the widest original wound margin was used for scoring. Sections which indicated abscess formacion were excluded from the evaluation. The parameters measured were wound closure, granulation tissue thickness, matrix density, number of infiltrated cells, and number of capillaries. Each of the parameters was graded numerically as described below

to permit average scores to be compiled for each parameter.

Wound Closure. - the degree of wound closure was measured and given a value of 0 to 10; 0 was equivalent to no closure and 10 was equivalent to complete closure by reepithelized keratinocytes.

Dermal Tissue Thickness.- A value of 1 equals a thin granulation, 2 equals moderate granulation, 3 equals a thick granulation, 4 equals a very thick granulation.

Matrix Density. - The degree of dermal matrix deposition was determined and scored as 1 equals edematous with little matrix, 2 equals a small amount of coarse matrix, 3 equals a moderate amount of matrix, 4 equals dense matrix.

Infiltrated Cells. - As an index of the degree of infiltrated cells, the number of fibroblasts and macrophages was estimated. Polymorphonuclear cells and lymphocyte were excluded from the counting. A score of 1 equals few cells, 2 equals a moderate number, 3 equals many cells, 4 equals very many cells.

Capillaries. - The number of mature capillaries was counted in the complete wound cross section at X100 magnification. A score of 0 equals 0-4 capillaries per wound, 1 equals 5-14 per wound, 2 equals 15-24 capillaries per wound, etc.

Tensile Strength

A full thickness, vertical dorsal incision (3 cm) was made with a scalpel. After the application of bFGF or the vehicle solution, the incision was closed using a monofilament Nylon suture (5-0, Ethicon Inc., Sommerville, NJ) placed at 1 cm intervals. Mice were sacrificed on day 9 post-wounding, sutures were removed, and three strips of skin (about 1 cm wide) were taken. The first strip was used for histological evaluation, and the other two strips were kept wet with PBS and used for fresh tensile strength measurements. A tensinometer was made by ourselves using a spring balance (Maximum 250g, Ohaus Scale Corp., Florham Park, NJ). A force was applied across the incision at a constant speed (1 cm/sec). The breaking strength was the point of maximal stress before wound separation, and was expressed as g/mm incisional width. Measurements were done in a blind way.

Results

Our early attempts to measure the effects of bFGF on full thickness wounds in normal mice such as BalbC and C57 BL indicated that the application of the growth factor had small positive effects, but dose effects were not confirmed (data not shown). As shown in Table I, heterozygous littermates had good wound closure rates and granulation tissue formation. The application of

bFGF to these mice did not promote significant wound healing although it had a small effect. db/db mice had impaired wound healing compared to their heterozygous litter mates (Table I). The differences in wounding healing were apparent primarily in the dermal parameters measured such as granulation tissue thickness, matrix density, infiltrated cells and capillary numbers. A slight difference between the two groups in wound closure was also observed in this experiment. When bFGF (5 ug) was applied to the wounds of db/db mice, a strong response was observed in all of the dermal parameters and a slight response occurred in the degree of wound closure. The addition of bFGF to wounds in db/db mice increased responses in the dermal parameters which made the degree of wound healing almost equal to that observed in the control heterozygous mice.

The dose of bFGF required to stimulate the increase in healing in db/db mice was next determined. Doses of 0.05, 0.5, and 5 ug were applied once a day for five days to wounds (Table III B). At 0.05 ug/day of bFGF, a small increase was seen in the number of infiltrated cells as well as the number of capillaries. At 0.5 ug/day of bFGF strong increases in all of the dermal parameters were apparent. In fact, the number of capillaries was significantly higher than that seen in mice receiving the lower dose. At 5 ug/day of bFGF a further increase was seen. Thus, the effective dose of

bFGF required for a significant increase in wound healing is between 0.05 and 0.5 ug/day when applied multiple times. However, none the doses tested had an effect on the degree of wound closure.

Since Davidson et al. reported that iodinated cartilage derived growth factor, which appears to be a form of bFGF, disappeared from wounds within 24 hr after injection, we next tested whether there were significant differences in healing between a single dose vs multiple doses of growth factor. Single dosing at 0.5 ug as well as 5 ug gave similar strong responses as multiple doses at 5 ug (Table III A).

The preparation of bFGF in methylcellulose might to increase the amount of growth factor retained at the local site because of its increased viscosity. However, application of bFGF in PBS generated the same response as bFGF in methylcellulose solution (Table III B). To insure the effects measured resulted from the bFGF, two additional experiments were conducted. Boiled bFGF at 5 ug/day did not show significant effect in increasing the number of infiltrated cells or capillary number (Table 3). Furthermore, bFGF pretreated with anti-bFGF IgG, which neutralized the effect of bFGF in vitro on bovine capillary endothelial cells, blocked the histological responses, but non-immune IgG did not (Table III B). These data clearly showed that bFGF itself is responsible for the responses measured.

In order to measure the effects of bFGF on wound healing in db/db mice as a function of time, we measured the effects of the growth factor at 5, 8, 12 and 18 days post-wounding. These results were compared to the healing in non-treated db/db wounds (Table IV). All of the parameters monitored in the bFGF-treated mice exceeded than those in non-treated mice. However, significant differences were not observed with respect to the rate of wound closure. All the granulation parameters, especially infitrated cell number and capillary number, showed significantly higher scores in bFGF-treated mice ($p < 0.01$). All of the granulation parameters in bFGF-treated mice continued to increase between 8 and 12 days. Interestingly, between 12 and 18 days, the granulation response appeared to begin to resolve. There was a decrease in the thickness of the granulation tissue, the number of infiltrated cells, and the number of capillaries. Meanwhile, matrix density continued to increase, which suggests matrix maturation and remodeling.

We next attempted to measure breaking strength in incisional wound to confirm that increased matrix formaiton contributed to wound strength. Figure 1 illustrates the results of tensile strength measurements in db/db mice and db/+ wound preparations. Healed wounds from db/db mice had only 56% of the tensile strength observed in healed wounds from db/+ litter mates.

Application of 5 ug bFGF in a single dose the strength of the wound 24% in normal littermates, and 46% in db/db mice. As a result, tensile strength in bFGF treated db/db wounds reached a level similar to that of wounds from non-treated heterozygous littermates. These data suggest that increased granulation tissue contributes to the strength of the wound site.

Discussion

These experiments provide evidence that recombinant bFGF is capable of significantly improving the degree of dermal healing in healing impaired mice. The observed increases in granulation tissue thickness, infiltrated cells, capillaries, and tensile strength are consistent with the proposal that bFGF stimulates the granulation response.

Unlike EGF, bFGF appeared to have little effect on wound closure. Since keratinolytes respond to bFGF (O'Keefe et al. 1988), this result was somewhat surprising. Perhaps because we used an open wound system, the presence of the large crust on the granulation tissue affected the migration of keratinocytes. Therefore, it will certainly be of interest to test the effect of bFGF on wounds with occlusive dressings.

It is also interesting that we could see only a small effect of bFGF on the rate or degree of healing in normal mice, though there are several reports

which demonstrated positive effects in normal rats (Davidson et al., 1985, McGee et al., 1988). Normal mice may have sufficient of growth factors and a normal wound healing system so that excess growth factor has no effect.

The effective dose of bFGF required for a significant increase in wound healing was determined to be 0.5 ug/day after multiple applications. A single dose of bFGF at 0.5 ug was also effective. The reason why a single dose was as effective as mutiple doses is not clear. Multiple doses of high concentrations of bFGF did not induce unlimited granulation tissue formation, and after wound closure a decrease of granulation tissue and induction of matrix occurred suggesting that bFGF can be used as a self-limited, potent wound healing potentiating agent.

References

1. Danon, D., M.A. Kowatch, and G.S. Roth. 1989. Promotion of wound repair in old mice by local injection of macrophages. Proc. Natl. Acad. Sci. USA 86:2018-2020.

2. Davidson J.M., M. Klagsbrun, K.E. Hill, A. Buckley, R. Sullivan, P.S. Brewer, and S.C. Woodward. 1985. Accelerated wound repair, cell proliferation, and collagen accumulation are produced by a cartilage-derived growth factor. J. Cell. Biol. 100: 1219-1227.

3. McGee, G.S., J.M. Davidson, A. Buckley, A. Sommer, S.C. Woodward, A.M. Aquino, R. Barbour, and A.A., Demetriou. 1988. Recombinant basic fibroblast growth factor accelerates wound healing. J. Sung Res. 45: 145-153.

4. O'Keefe, E.J., M. L. Chiu, and R.E. Payne. 1988. Stimulation of

growth of Keratinocytes by basic fibroblast growh factor. J. Invest Dermatol. 90: 767-769.

5. Pierce, G.F., T.A. Mustoe, R.M. Senior, J. Reed, G.L. Griffin, A. Thomason, and T.F. Deuel. 1988. In vivo incisional wound healing augmented by platelet-derived growth factor and recombinant c-sis gene homodimic proteins. J. Exp. Med. 167: 974-987.

6. Reed, B.R., and R.A.F. Clark. 1985. Cutaneous tissue repair: Practical implications of current knowledge. II. J. AM. Acad. Dermatol. 13: 919-941.

TABLE I

Effects of bFGF on Wound Healing in db/db Mice and Normal Littermates

Treatment	N	Wound Closure	Granulation Tissue	Matrix Density	Infiltrated Cells	Capillary Number
Normal Mice (db/+) 0 ug x 5 days	10	8.8 ± 0.6	3.3 ± 0.1	3.3 ± 0.3	3.2 ± 0.2	8.1 ± 0.8
Normal Mice (db/+) 5 ug x 5 days	9	8.6 ± 0.7	3.6 ± 0.2	3.4 ± 0.3	3.8 ± 0.1	10.6 ± 1.3
db/db Mice, 0 ug x 5 days	10	7.1 ± 0.7	1.4 ± 0.2	2.1 ± 0.4	1.7 ± 0.1	2.4 ± 0.4
db/db Mice 5 ug x 5 days	10	8.4 ± 0.6	2.8 ± 0.2	3.1 ± 0.2	2.8 ± 0.2	8.2 ± 0.9

Samples were taken at 8 days post-wounding. Vehicle solutions plus and minus bFGF were applied each day for five days beginning with the day of wounding.

TABLE II

Dose Effect on Wound Healing in Normal and db/db Mice

A. db/+ mice

Sample	Percentage of Wound Closure	Epidermal Thickness	Dermal Thickness	Compactness of Dermis	Infiltrated Cells	Capillary Number
0 ng X 5 days (11)	6.9 ± 0.8	0.7 ± 0.4	2.6 ± 0.2	3.5 ± 0.3	3.1 ± 0.2	8.8 ± 1.2
50 ng X 5 days (10)	7.8 ± 0.9	1.2 ± 0.3	2.4 ± 0.2	3.2 ± 0.2	2.9 ± 0.2	7.2 ± 1.1
500 ng X 5 days (8)	7.0 ± 0.9	0.8 ± 0.3	3.3 ± 0.3	3.3 ± 0.3	3.4 ± 0.2	8.4 ± 1.6
5000 ng X 5 days (8)	9.6 ± 0.4	2.3 ± 0.3	3.1 ± 0.3	3.5 ± 0.2	3.1 ± 0.3	6.9 ± 1.3

TABLE II (Cont'd)

Dose Effect on Wound Healing in db/db Mice

B. db/db Mice

Treatment	N	Percentage of Wound Closure	Granulation Tissue Thickness	Matrix Density	Infiltrated Cells	Capillary Number
0 ug x 5 days	10	5.0 ± 0.6	1.6 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	4.2 ± 1.0
0.05 ug x 5 days	10	5.0 ± 0.7	1.7 ± 0.1	1.9 ± 0.2	2.6 ± 0.3	6.8 ± 1.2
0.5 ug x 5 days	5	5.6 ± 1.1	2.8 ± 0.2	2.2 ± 0.3	3.0 ± 0	12.8 ± 1.4
5 ug x 5 dats X 5 days (8)	8	4.3 ± 0.9	3.0 ± 0.3	3.6 ± 0.2	3.8 ± 0.2	13.8 ± 2.9

Samples were taken at 8 days post-wounding. Solutions were applied once a day each day for five days beginning with the day of wounding.

TABLE III A

Comparison of Wound Healing in db/db Mice with Single vs Multiple Doses of bFGF

Treatment	Percentage of Wound Closure	Epidermal Thickness	Dermal Thickness	Compactness Thickness	Infiltrated of Dermis	Capillary No.
0 (7)	4.0 ± 1.0	0	1.8 ± 0.2	1.5 ± 0.3	1.5 ± 0.3	5.5 ± 1.6
5000 ng	5.3 ± 0.8	0	2.0 ± 0.3	2.1 ± 0.3	2.3 ± 0.2	8.8 ± 1.5
X 1 day (8)						
5000 ng	5.8 ± 1.1	0.5 ± 0.3	2.4 ± 0.2	2.9 ± 0.1	2.8 ± 0.2	9.3 ± 1.2
X 5 days (8)						

Samples taken at 8 days post-wounding. The number of slides examined in each group is indicated by the number in parenthesis. Vehicle solutions plus and minus bFGF were applied beginning with the day of wounding.

TABLE III B

Effects of bFGF on Wound Healing in (db/db) Mice

Treatment	N	Wound Closure	Granulation Tissue Thickness	Matrix Density	Infiltrated Cells	Capillary Number
0.05 ug x 1 day	8	7.5 ± 0.8	1.6 ± 0.2	1.9 ± 0.2	1.9 ± 0.3	7.5 ± 1.5
0.5 ug x 1 day	10	6.4 ± 0.7	2.5 ± 0.2	2.5 ± 0.2	2.5 ± 0.2	12.1 ± 1.1
5 ug in MC x 1 day	8	8.9 ± 0.5	2.8 ± 0.2	2.8 ± 0.2	2.9 ± 0.1	13.5 ± 1.3
0 ug x 5 days	9	6.1 ± 0.9	1.9 ± 0.1	2.1 ± 0.3	1.8 ± 0.1	5.2 ± 0.6
5 ug x 5 days	10	5.3 ± 0.7	2.5 ± 0.2	2.2 ± 0.2	2.6 ± 0.2	12.9 ± 1.8
5 ug in PBS x 5 days	8	6.3 ± 0.8	2.5 ± 0.2	2.6 ± 0.2	2.6 ± 0.2	10.1 ± 1.0
Boiled 5 ug x 5 days	9	6.2 ± 0.6	1.6 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	7.1 ± 0.9
0.5 ug + anti-bFGF IgG x 1 day	7	7.0 ± 1.0	2.1 ± 0.1	2.0 ± 0	2.1 ± 0.1	7.9 ± 1.6
0.5 ug + non-immune IgG x 1 day	7	6.7 ± 0.9	2.9 ± 0.1	2.9 ± 0.2	2.9 ± 0.2	14.3 ± 2.1

Samples taken at 8 days post-wounding.

TABLE IV

Time Course of Wound Healing in db/db Mice With and Without bFGF.

Treatment	N	Wound Closure	Granulation Tissue Thickness	Matrix Density	Infiltrated Cells	Capillary Number
0 ug x 5 days	10	1.7 ± 0.2	1.3 ± 0.1	1.0 ± 0	1.3 ± 0.1	1.8 ± 0.6
8 days	9	3.6 ± 0.6	1.2 ± 0.1	1.1 ± 0.1	1.4 ± 0.2	3.4 ± 1.3
12 days	9	7.7 ± 0.6	2.0 ± 0.2	2.3 ± 0.2	2.1 ± 0.2	5.8 ± 0.8
18 days	11	10.0 ± 0	1.7 ± 0.2	3.0 ± 0.2	2.0 ± 0.2	7.0 ± 0.9
5 ug x 5 days	10	2.0 ± 0.2	1.3 ± 0.1	1.0 ± 0	1.4 ± 0.2	3.4 ± 1.1
8 days	8	3.8 ± 0.7	2.6 ± 0.2	2.1 ± 0.2	2.6 ± 0.2	11.9 ± 2.7
12 days	10	7.9 ± 0.9	2.9 ± 0.3	3.2 ± 0.2	3.2 ± 0.2	16.8 ± 1.9
18 days	10	10.0 ± 0	2.3 ± 0.1	3.6 ± 0.2	2.5 ± 0.2	10.4 ± 0.9

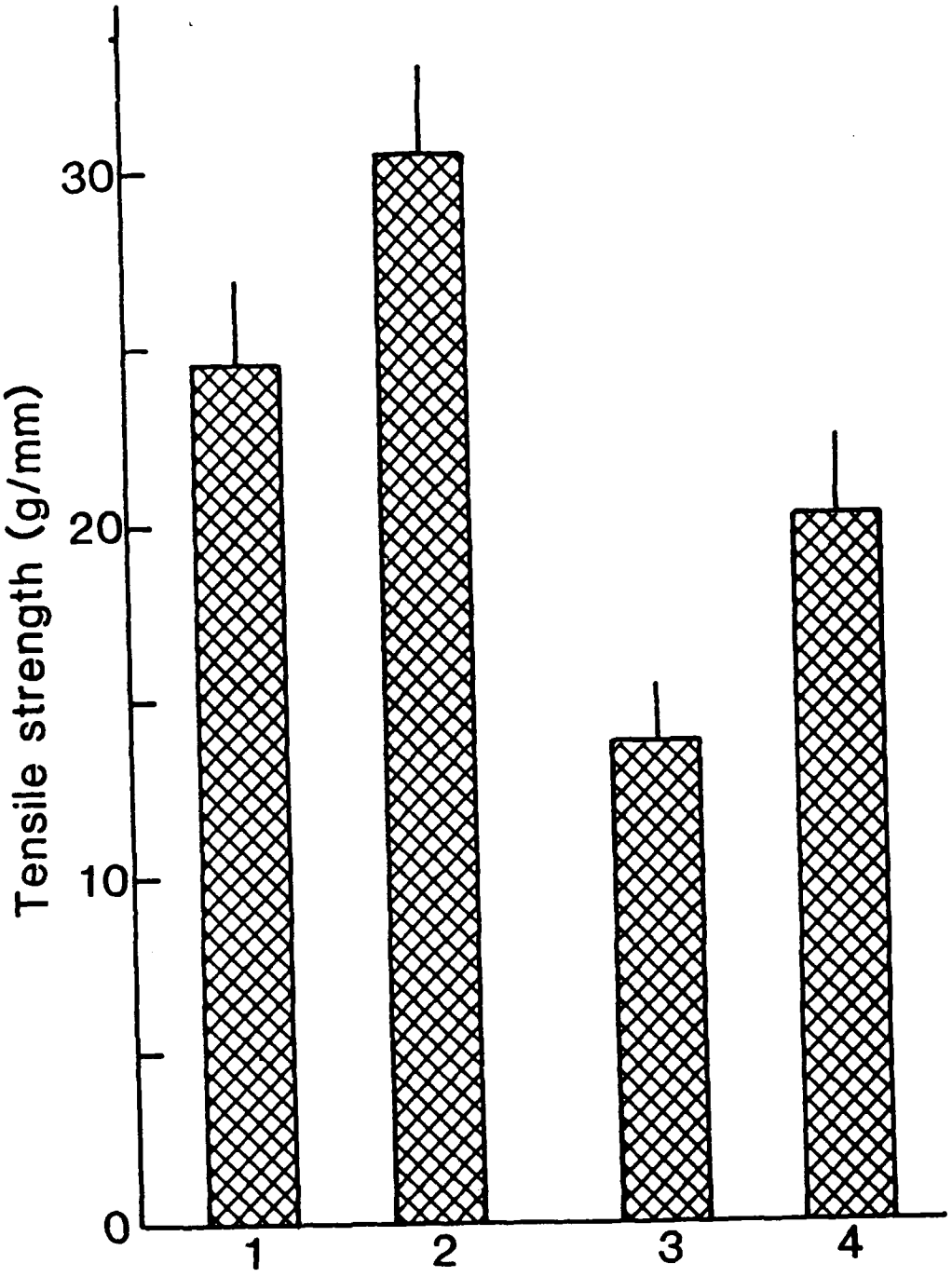

Figure 1 Tensile strength of normal and db/db wounds. Wounds were made in db/db mice and heterozygous littermates as described in Methods. Animals were treated with .5 ug/wound of bFGF or used as controls. The wounds were then prepared and tested as described in Methods. 1, db/+ control; 2, db/+ plus 5 ug bFGF; 3, db/db, control; 4, db/db plus 5 ug bFGF.